REMARKS

Claims 14, 16-20, and 28-35 are pending in the application, with 14 and 29 being the independent claims. Claim 20 has been withdrawn by the Examiner. Based on the following remarks, Applicants respectfully request that the Examiner reconsider all outstanding objections and rejections and that they be withdrawn.

Rejections Under 35 U.S.C. § 103

Claims 14, 16-19, and 28-35 have been rejected under 35 U.S.C. § 103(a) as allegedly being unpatentable over Trinchieri *et al.* (U.S. Patent No. 6,375,944). (Office Action, page 3). Applicants respectfully traverse this rejection.

The Examiner is of the opinion that Trinchieri *et al.* teaches the administration of an immunogen with compound 48/80 to a subject to induce an immune response in the subject. (Office Action, page 3). The Examiner's opinion is based on the idea that Trinchieri *et al.* teaches the use of calmidazolium and that calmidazolium and compound 48/80 are the same compound having the same CAS number, 94724-12-6. (Office Action, page 3).

Applicants respectfully disagree. Trinchieri *et al.* discloses methods for enhancing the therapeutic or adjuvant effects of interleukin-12 by co-administering a nitric oxide inhibiting and/or neutralizing agent (column 6, lines 17-33). In one embodiment, the nitric oxide inhibiting and/or neutralizing agent can be calmidazolium (column 10, line 1). Trinchieri *et al.* is silent regarding compound 48/80.

Applicants respectfully assert that the Examiner is mistaken regarding the identity of compound 48/80 and calmidazolium. These are two very different products that are not related to each other in any way. This is evidenced by the pages from the Sigma-Aldrich catalog (attached hereto as Exhibit A), which show two separate listings for the products. According to the Sigma-Aldrich catalog, compound 48/80 (CAS Number 94724-12-6) is a mixture of low-molecular weight polymers of p-methoxyphenethyl methylamine with formaldehyde having a degree of polymerization between 3 to 6. In contrast, calmidazolium (CAS Number 57265-65-3) is a single compound comprising an imidazole group and several chlorophenyl groups. The distinction between the two compounds is confirmed in the product data sheets from the Enzo Life Sciences catalog (attached hereto as Exhibit B), which assign the same structures and CAS Numbers as in the Sigma-Aldrich catalog. Further

confirmation that compound 48/80 and calmidazolium are completely distinct products is found in Gietzen (*Biochem. J. 216*:611 (1983) (Exhibit C)) and Tuana and MacLennan (*J. Biol. Chem. 259*:6979 (1984) (Exhibit D)), each of which describes experiments comparing the biological activities of compound 48/80 and calmidazolium.

Thus, the Examiner is incorrect regarding the identity of compound 48/80 and calmidazolium. As Trinchieri *et al.* fails to disclose compound 48/80 or its use as an adjuvant and the disclosure of calmidazolium is irrelevant, the present claims cannot be considered obvious over the cited reference.

It is respectfully requested that the rejection of claims 14, 16-19, and 28-35 under 35 U.S.C. § 103(a) be withdrawn.

CONCLUSION

Accordingly, Applicant submits that the present application is in condition for allowance and the same is earnestly solicited. The Examiner is encouraged to telephone the undersigned at 919-854-1400 for resolution of any outstanding issues.

Respectfully submitted,

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CERTIFICATION OF TRANSMISSION

I hereby certify that this correspondence is being transmitted via the Office electronic filing system in accordance with § 1.6(a)(4) to the U.S. Patent and Trademark Office on October 2, 2009.

Signature: ______________________
Marthenn Salazar

EXHIBIT A

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SIGMA®

ProductInformation

Compound 48/80

Product Number **C 2313**
Storage Temperature -0 °C

Product Description
Molecular Formula: $C_{11}H_{15}NO$ (monomer)[2]
Molecular weight: 153 (monomer)[2]
CAS Number: 94724-12-6

Compound 48/80 is a condensation product of p-methoxyphenethyl methylamine with formaldehyde; it is a mixture of low-molecular weight polymers having a degree of polymerization between 3 to 6.[1]

Compound 48/80 is a potent histamine releasing agent, primarily from mast cells, with a subsequent depletion of tissue histamine. It is the action of mast cell mediators on the cardiovascular system that leads to circulatory collapse.[3] The toxicity is due to more than histamine release.[5] It is a potent inhibitor of phospholipase C.[6]

This material has been used to induce degranulation of GnRH-like immunoreactivity of mast cells in the brain and mesentery[7] and for elucidating the mechanism by which anti-allergic medications suppress conjunctivitis.[8]

Precautions and Disclaimer
For Laboratory Use Only. Not for drug, household or other uses.

Preparation Instructions
This product is soluble in water (50 mg/ml), yielding a clear solution.

Storage/Stability
Solutions can be autoclaved at 15 psi for 30 minutes with no detectable change in toxicity or potency.

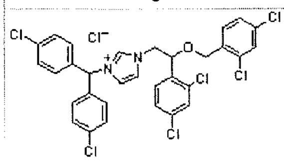
References
1. Lagunoff, D., et al., Agents that release histamine from mast cells. Annu. Rev. Pharmacol. Toxicol., **23**, 331-351 (1983).
2. Baltzley, R., et al., A family of long-acting depressors. J. Am. Chem. Soc., **71**, 1301-1305 (1949).
3. Niemegeers, C. J., et al., Protection of rats from compound 48/80-induced lethality. A simple test for inhibitors of mast cell-mediated shock. Arch. Int. Pharmacodyn. Ther., **234(1)**, 164-176 (1978).
4. Read, G. W., and Lenney, J. F., Molecular weight studies on the active constituents of compound 48-80. J. Med. Chem., **15(3)**, 320-323 (1972).
5. Papacostas, et al., Arch. Int. Pharmacodyn. Ther., **120**, 353 (1959).
6. Bronner, C., et al., Compound 48/80 is a potent inhibitor of phospholipase C and a dual modulator of phospholipase A2 from human platelet. Biochim. Biophys. Acta, **920(3)**, 301-305 (1987).
7. Yang, M. F., et al., Compound 48/80-induced degranulation of GnRH-like immunoreactive mast cells in the brain and mesentery of gerbil. Zoological Studies, **41(1)**, 99-110 (2002).
8. Li, Q., et al., Suppressive effect of antiflammin-2 on compound 48/80-induced conjunctivitis. Role of phospholipase A2s and inducible nitric oxide synthase. Ocul. Immunol. Inflamm., **6(2)**, 65-73 (1998).

AGW/RXR 5/03

C3930 Calmidazolium chloride

Sigma solid

Image



1 of 1

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- Enzyme Inhibitors by Enzyme > A to C > Calmodulin-regulated enzymes
- Cancer Research > Tumor Growth Regulation > PI3K-Akt-mTOR Enzyme Regulators

Last 5 Products Viewed
- C3930 (Sigma)
- C278 (Sigma)

Price and Availability

Product Number	Your Price USD	Available to Ship	Quantity	Actions
C3930-5MG	109.50	08/03/2009 details...		
C3930-10MG	182.50	08/03/2009 details...		
C3930-25MG	438.50	08/03/2009 details...		
C3930-50MG	658.00	08/03/2009 details...		

Synonym:	R 24571, 1-[bis(4-Chlorophenyl)methyl]-3-[2-(2,4-dichlorophenyl)-2-(2,4-dichlorobenzyloxy)ethyl]-1H-imidazolium chloride, 1-[Bis(4-chlorophenyl)methyl]-3-[2,4-dichloro-β-(2,4-dichlorobenzyloxy)phenethyl] imidazolium chloride
CAS Number:	57265-65-3
Linear Formula:	$C_{31}H_{23}Cl_7N_2O$
Molecular Weight:	687.70
Beilstein Registry Number:	6470244
MDL number:	MFCD00077679
PubChem Substance ID:	24278023

Specifications | Related Products | References | Reviews

Description

Biochem/physiol Actions	Inhibitor of calmodulin-regulated enzymes
Caution	Binds to glass surfaces in aqueous solutions.
Reconstitution	Stock solutions may be prepared in DMSO at 5 mM then diluted to 0.01 mM with buffer. DMSO solutions may be stored for several weeks.

Properties

form	solid
color	white
storage temp.	2-8°C

Safety

Safety Statements	22-24/25
WGK Germany	3

EXHIBIT B

PRODUCT DATA SHEET

Enzo® Life Sciences

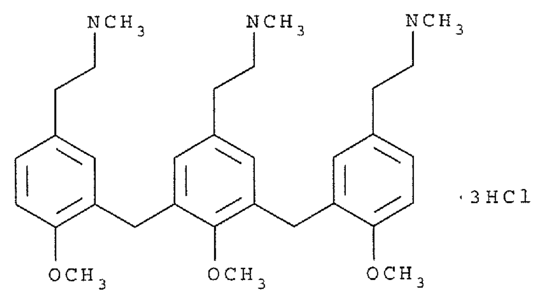
PRODUCT: Compound 48/80

CAS NO: 94724-12-6

CATALOG NO.: EI-179

LOT#: temp

STRUCTURE:



PHYSICAL APPEARANCE: white solid

MOLECULAR FORMULA: See PURITY.

AVG. MOL. WEIGHT: 630.0

PURITY: Compound 48/80 is an oligomeric mixture of condensation products of N-methyl-p-methoxyphenethylamine and formaldehyde. The trimer is the most abundant oligomer of the mixture.

SOLUBILITY: Soluble in water (100mg/ml)

STORAGE: Store, as supplied, at 0-4°C for up to 1 year. Store solutions at -20°C for up to 3 months.

APPLICATION NOTES: Activates G proteins by a mechanism analogous to that of mastoparan.[1,2] Inhibits calmodulin[3,4] and human platelet PLC and exhibits a concentration dependent biphasic modulation of human platelet PLA_2[5].

REFERENCES:
1. T.Higashijima *et al.J.Biol.Chem.* 1990 **265** 14176
2. M.Mousli *et al. FEBS Lett.* 1990 **259** 260
3. K.Gietzen *et al. Biochim.Biophys.Acta* 1983 **736** 109
4. K.Gietzen *Biochem.J.* 1983 **216** 611
5. C.Bronner *et al. Biochim.Biophys.Acta* 1987 **920** 301

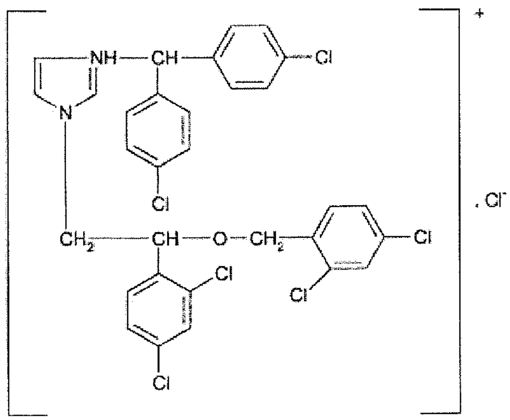
The pharmacological and toxicological properties of this product have not been fully investigated. Exercise caution in use and handling. This product must not be used in humans.

PRODUCT DATA SHEET

ALX-430-026

Calmidazolium chloride

[1-[*bis*(p-Chlorophenyl)methyl]-3-[2,4-dichloro-β-(2,4-dichlorobenzyloxy)phenethyl] imidazolium chloride]



Product Number/Sizes

ALX-430-026-M010	10 mg
ALX-430-026-M050	50 mg

Product Specifications

FORMULA:	$C_{31}H_{23}Cl_7N_2O$
MW:	687.7
CAS NUMBER:	57265-65-3
PURITY:	≥98%
APPEARANCE:	White powder.
SOLUBILITY:	Soluble in methanol, 100% ethanol, chloroform, DMSO or propylene glycol; almost insoluble in water.
SHIPPING:	AMBIENT
LONG TERM STORAGE:	+4°C

Product Description

Calmodulin antagonist. At least 150 times more potent than trifluoperazine (Prod. No. ALX- 550-310) as an inhibitor of brain calmodulin-dependent phosphodiesterase. Inhibitor of voltage-gated Ca^{2+} channels. Blocks Ca^{2+}-calmodulin binding to NOS.

Product Specific Literature References

Inhibitors of calmodulin impair the constitutive but not the inducible nitric oxide synthase activity in the rat aorta: V.B. Schini & P.M. Vanhoutte; J. Pharmacol. Exp. Ther. **261**, 553 (1992)
Calmidazolium, a calmodulin inhibitor, inhibits endothelium-dependent relaxations resistant to nitro-L-arginine in the canine coronary artery: S. Illiano, et al.; Br. J. Pharmacol. **107**, 387 (1992)
The calmodulin antagonist calmidazolium stimulates release of nitric oxide in neuroblastoma N1E-115 cells: J. Hu & E.E. el-Fakahany; Neuroreport **4**, 198 (1993)
Vasoactive intestinal peptide/pituitary adenylate cyclase-activating peptide-dependent activation of membrane-bound NO synthase in smooth muscle mediated by pertussis toxin-sensitive Gi1-2: K.S. Murthy & G.M. Makhlouf; J. Biol. Chem. **269**, 15977 (1994)
Mitochondrial nitric oxide synthase is constitutively active and is functionally upregulated in hypoxia: Z. Lacza, et al.; Free Radic. Biol. Med. **31**, 1609 (2001)
Mitochondrial calcium uptake stimulates nitric oxide production in mitochondria of bovine vascular endothelial cells: E.N. Dedkova, et al.; Am. J. Physiol. Cell Physiol. **286**, C406 (2004)
Neural tube closure depends on nitric oxide synthase activity: A. Nachmany, et al.; J. Neurochem. **96**, 247 (2006)

Revised 01-Feb-09

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EXHIBIT C

Biochem. J. (1983) **216**, 611–616
Printed in Great Britain

Comparison of the calmodulin antagonists compound 48/80 and calmidazolium

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(*Received 14 June 1983/Accepted 23 August 1983*)

The two presumed calmodulin antagonists calmidazolium and compound 48/80 were compared for their effects on several calmodulin-dependent and calmodulin-independent enzyme systems. Compound 48/80 and calmidazolium were found to be about equipotent in antagonizing the calmodulin-dependent fraction of brain phosphodiesterase and erythrocyte Ca^{2+}-transporting ATPase. Compound 48/80 combines high potency with high specificity in that: (1) the basal, calmodulin-independent, activity of calmodulin-regulated enzymes was not suppressed; (2) calmodulin-independent enzyme activities, such as Ca^{2+}-transporting ATPases of sarcoplasmic reticulum, Mg^{2+}-dependent ATPases of different tissues and Na^{+}/K^{+}-transporting ATPase of cardiac sarcolemma, were far less altered, or not altered at all, by compound 48/80 as compared with calmidazolium; and (3) antagonism of proteolysis-induced stimulation as opposed to calmodulin-induced activation of erythrocyte Ca^{2+}-transporting ATPase required a 32 times higher concentration of compound 48/80. In all these aspects compound 48/80 was found to be a superior antagonist to calmidazolium since inhibition of calmodulin-independent events by the other agent occurred at considerably lower concentrations. Therefore compound 48/80 is proposed to be a much more specific and useful tool for studying the participation of calmodulin in biological processes than the presently used agents.

Calmodulin, the ubiquitous Ca^{2+}-dependent regulatory protein, plays a pivotal role in all eukaryotic cells. It confers Ca^{2+}-sensitivity on a multitude of enzyme systems and cell functions. Its properties and functions have been summarized in recent reviews (Cheung, 1980; Klee *et al.*, 1980; Means & Dedman, 1980).

The calmodulin-dependent fraction of these enzymes can be inhibited by a wide range of chemically unrelated substances, such as phenothiazines and butyrophenones (Levin & Weiss, 1976; Gietzen *et al.*, 1980), naphthalene sulphonamides (Kobayashi *et al.*, 1979), Vinca alkaloids (Watanabe *et al.*, 1979; Gietzen & Bader, 1980), local anaesthetics (Volpi *et al.*, 1981), calmidazolium (Gietzen *et al.*, 1981), formerly referred to as R 24571, and compound 48/80 (Gietzen *et al.*, 1983). Several calmodulin inhibitors were also shown to antagonize the enzymes' activity stimulated by treatments mimicking the action of calmodulin on phosphodiesterase and Ca^{2+}-transporting ATPase (Wolff & Brostrom, 1976; Gietzen *et al.*, 1982*a*). In a recent study a general model has been proposed illustrating the molecular mechanism of activation and inhibition of calmodulin-regulated enzymes simply by the assumption of hydrophobic and ionic interactions (Gietzen *et al.*, 1982*a*). Generally, activators of calmodulin-dependent enzymes (calmodulin, oleic acid or phosphatidylserine) can be considered as anionic amphiphiles, whereas calmodulin antagonists are cationic amphiphiles at physiological pH.

Evidence has been presented that inhibition of a calmodulin-stimulated enzyme may occur according to the following modes: (1) calmodulin is complexed by the cationic amphiphilic antagonist, as a result of their complementary structural features, via ionic and hydrophobic interactions (Weiss *et al.*, 1980); and (2) in addition, several calmodulin antagonists exert their inhibitory effect via direct interaction with the calmodulin effector enzyme (Gietzen *et al.*, 1982*a*,*b*). Therefore, these substances cannot be considered as calmodulin-specific probes. Moreover, almost all described inhibitors are more or less unspecific in that they also inhibit the basal activity of calmodulin-dependent enzymes and even the activity of calmodulin-independent enzymes (Balzer *et al.*, 1968; Levin & Weiss, 1976;

Kobayashi *et al.*, 1979; Gietzen & Bader, 1980; Gietzen *et al.*, 1980, 1981; Luthra, 1982).

From the numerous calmodulin antagonists two were shown to be outstanding. (1) Calmidazolium was reported to be the most potent inhibitor (Gietzen *et al.*, 1981; Van Belle, 1981) and in addition the substance displayed a higher specificity for calmodulin-induced activation of phosphodiesterase and Ca^{2+}-transporting ATPase compared with other modes of stimulation (Gietzen *et al.*, 1982*a*). (2) Compound 48/80 was shown to be the most specific antagonist of calmodulin-dependent Ca^{2+}-transporting ATPase activity as opposed to basal, calmodulin-independent, ATPase activity (Gietzen *et al.*, 1983).

In the present paper calmidazolium and compound 48/80 were compared directly under identical experimental conditions. The effects of both antagonists on several Ca^{2+}–calmodulin-dependent, Ca^{2+}-dependent but calmodulin-independent and Ca^{2+}–calmodulin-independent enzymes were investigated. In nearly all respects, compound 48/80 proved to be superior to calmidazolium.

Materials and methods

All reagents were of the highest purity available. Calmidazolium was supplied by Janssen Pharmaceutica (Beerse, Belgium). Compound 48/80 (product no. C4257), oleic acid, 5′-nucleotidase and soya-bean trypsin inhibitor were obtained from Sigma (München, Germany).

The lipophilic compound calmidazolium was dissolved in dimethyl sulphoxide and added to the respective assay medium with vigorous mixing. The final concentration of dimethyl sulphoxide in the assay media including the controls was always 1% (v/v).

Oleic acid microdispersions were prepared by sonication (Branson Sonifier B12; approx. 2 min at setting 2) in a buffer containing 0.1 mM-EGTA and 5 mM-4-morpholinepropanesulphonic acid (Mops, pH 7.0) under a stream of N_2.

Preparation of enzymes and calmodulin

Homogeneous calmodulin was prepared from bovine brain as described by Kakiuchi *et al.* (1981). Calmodulin-sensitive phosphodiesterase was partially purified, based on the method of Wang & Desai (1977) and slightly modified as described by Gietzen *et al.* (1982*a*). Human erythrocyte membranes deficient in calmodulin were prepared by the procedure of Gietzen & Kolandt (1982). Sarcoplasmic-reticulum vesicles were prepared from dog heart by the procedure of Suko & Hasselbach (1976) and from rabbit skeletal muscle by the procedure described by Meissner *et al.* (1973). Vesicles of calf cardiac sarcolemma were purified as reported by Jones *et al.* (1979) and Caroni *et al.* (1980).

Controlled tryptic digestion

Ca^{2+}-transporting ATPase was digested at 37°C by 0.2 mg of trypsin/mg of erythrocyte membrane protein. Proteolysis was performed in the assay medium and was terminated with a 5-fold excess (w/w) of trypsin inhibitor (see Gietzen *et al.*, 1982*a*).

Assay of enzyme activities

ATPase and phosphodiesterase activities were determined at 37°C by measuring the rate of P_i-liberation as reported by Stewart (1974), slightly modified as described by Lanzetta *et al.* (1979).

Briefly, phosphodiesterase activity was assayed by coupling the phosphodiesterase reaction with 5′-nucleotidase reaction (Butcher & Sutherland, 1962; Wang & Desai, 1977) and measuring the P_i produced within 30 min. The assay mixture (final volume, 1 ml) consisted of 40 mM-Tris/HCl (pH 7.5), 40 mM-imidazole, 3 mM-magnesium acetate, 1.2 mM-cyclic AMP and 0.1 mM-$CaCl_2$.

The reaction of the different ATPases was followed discontinuously over various time periods depending on the specific activity of the respective enzyme. The assay medium for Ca^{2+}-transporting ATPase contained, in a final incubation medium of 1 ml, 25 mM-4-morpholinepropanesulphonic acid (pH 7.0), 100 mM-KCl, 0.25 mM-ouabain, 10 mM-NaN_3, 1 mM-ATP, 2 mM-$MgCl_2$ and a 0.2 mM-Mg^{2+}/0.2 mM-Ca^{2+}/0.4 mM-EDTA buffer to yield a free Ca^{2+} concentration of 36 μM (Wolf, 1973). Ca^{2+}-free controls contained, instead of the Mg^{2+}/Ca^{2+}/EDTA buffer, 0.2 mM-Mg^{2+}/0.4 mM-EGTA. These controls yielded simultaneously the Mg^{2+}-dependent ATPase activity. Ca^{2+}-transporting ATPase activity refers to the difference in activity obtained in the presence and in the absence of Ca^{2+}. The medium for Na^+/K^+-transporting ATPase consisted of 100 mM-NaCl, 10 mM-KCl, 30 mM-imidazole/HCl (pH 7.2), 4 mM-$MgCl_2$, 10 mM-NaN_3, 0.5 mM-Tris/EGTA and 2 mM-ATP, with or without 0.25 mM-ouabain. Na^+/K^+-transporting ATPase activity is defined as the difference in activity obtained in the presence and in the absence of ouabain.

To facilitate comparison, all enzyme assays were performed at the same protein concentration of 30 μg/ml unless otherwise stated. Before the reaction was started with the respective substrate (ATP, cyclic AMP) enzymes were pre-incubated as follows: Mg^{2+}-dependent ATPases, Na^+/K^+-transporting ATPase, sarcoplasmic-reticulum Ca^{2+}-transporting ATPases and tryptically digested erythrocyte Ca^{2+}-transporting ATPase were pre-incubated with the corresponding drug for 10 min at 37°C. Phosphodiesterase and erythrocyte Ca^{2+}-transporting ATPase were first pre-incubated for 10 min with the

drug and additionally for 10 min in the presence or in the absence of an activator.

Results

The stimulation of erythrocyte Ca^{2+}-transporting ATPase and brain phosphodiesterase by calmodulin and antagonism of the activation by compound 48/80 and calmidazolium is demonstrated in Figs. 1(*a*) and 1(*b*). In the absence of the agents, as documented by the points on the ordinate (Fig. 1*a*), Ca^{2+}-transporting ATPase of disrupted erythrocyte membranes could be maximally stimulated by calmodulin 5–6-fold above the basal enzyme's activity ('basal' Ca^{2+}-transporting ATPase activity was defined as that activity determined in the absence of added calmodulin). Phosphodiesterase displayed a somewhat higher sensitivity towards calmodulin in that calmodulin stimulated the enzyme in the absence of drugs 6.5–8-fold above its basal activity (Fig. 1*b*).

As can be seen from Fig. 1(*a*) calmidazolium antagonized the calmodulin-induced activation of erythrocyte Ca^{2+}-transporting ATPase with an IC_{50} value (concentration producing 50% inhibition) of $0.35\,\mu$M. However, also the calmodulin-independent activity of the enzyme was inhibited by this drug at higher concentrations ($IC_{50}=9\,\mu$M). On the other hand compound 48/80 specifically antagonized the calmodulin-dependent fraction of erythrocyte Ca^{2+}-transporting ATPase activity with an IC_{50} value of $0.85\,\mu$g/ml, whereas the basal activity was not at all affected at concentrations $\leqslant 300\,\mu$g/ml (Fig. 1*a*).

Both calmidazolium and compound 48/80 inhibited the calmodulin-stimulated fraction of rat brain phosphodiesterase with high potency and IC_{50} values of $0.15\,\mu$M and $0.3\,\mu$g/ml respectively (Fig. 1*b*). Again calmidazolium also antagonized the basal activity of this enzyme ($IC_{50}=20\,\mu$M). As was shown for Ca^{2+}-transporting ATPase, the basal activity of phosphodiesterase could not be suppressed by compound 48/80. This agent had the opposite effect on basal phosphodiesterase activity in that it slightly stimulated the enzyme activity above its basal level in the concentration range of 100–300 μg/ml (Fig. 1*a*).

The two drugs were also tested for their effects on two Ca^{2+}-transporting ATPases of sarcoplasmic reticulum of different tissues, being not calmodulin-dependent, at least not directly. Ca^{2+}-transporting ATPase of dog cardiac sarcoplasmic reticulum was inhibited half-maximally by calmidazolium at $2.1\,\mu$M and by compound 48/80 at $16\,\mu$g/ml (Table 1). Half-maximal inhibition of skeletal muscle sarcoplasmic-reticulum Ca^{2+}-transporting ATPase by calmidazolium occurred at $2.9\,\mu$M, whereas a rather high concentration ($80\,\mu$g/ml) of compound 48/80 was required to give the same effect (Table 1).

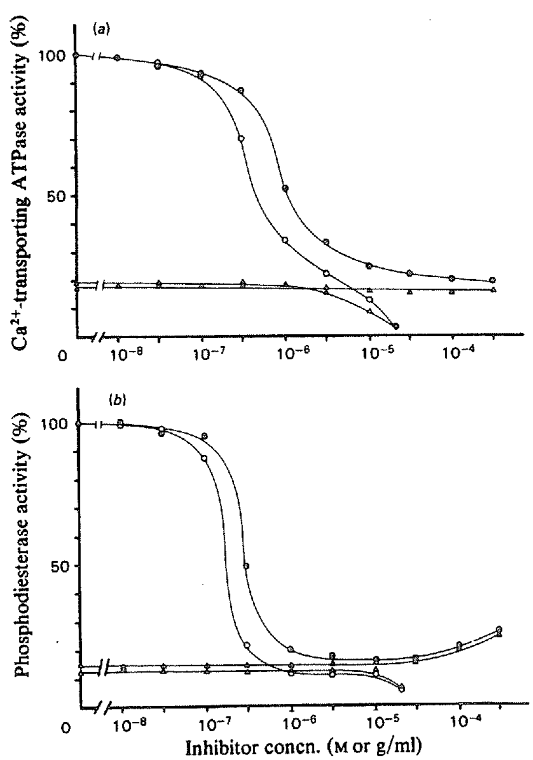

Fig. 1. *Effects of compound 48/80 and calmidazolium on erythrocyte Ca^{2+}-transporting ATPase (a) and rat brain phosphodiesterase (b)*

Basal, calmodulin-independent (▲ and △), and calmodulin (30 nM)-activated (● and ○) enzyme activities were determined in the absence and in the presence of various concentrations of compound 48/80 (● and ▲) and calmidazolium (○ and △). Note that the concentrations of compound 48/80 are given as g per ml and those of calmidazolium as M. Ca^{2+}-transporting ATPase (100% activity = 70–85 nmol/min per mg of protein) and phosphodiesterase (100% activity = 0.9–1 μmol/min per mg of protein) activities are related to the calmodulin-stimulated enzymes in the absence of drug. Each point represents the mean of four to six determinations.

In addition, the effects of both calmodulin antagonists on several Ca^{2+}–calmodulin-independent enzymes were investigated. Table 1 gives evidence that calmidazolium half-maximally antagonized calf cardiac sarcolemma Na^+/K^+-transporting ATPase at $15\,\mu$M. However, compound 48/80 at the highest concentration used ($300\,\mu$g/ml) inhibited this enzyme only by 25%. Calmidazolium proved also to possess inhibitory potency against Mg^{2+}-dependent ATPases of rabbit skeletal muscle sarcoplasmic reticulum and human erythrocytes with observed IC_{50} values of 3.3 and $20\,\mu$M

Table 1. *Effects of calmidazolium and compound 48/80 on different enzyme activities*
Enzyme activities were determined as described in the Materials and methods section at a concentration of 30μg of protein/ml, except for the cases marked *, in which the protein concentration was 60μg/ml. The IC_{50} values were obtained graphically from dose–effect curves. The coefficients of specificity represent the factors by which the inhibitors are more specific in antagonizing the calmodulin-dependent fraction of erythrocyte Ca^{2+}-transporting ATPase activity as compared with the listed enzyme activities, or, vice versa, the numbers give the factors by which the inhibitor concentrations were found to be higher in order to achieve half-maximal inhibition of the respective enzyme activities as opposed to the calmodulin-dependent activity. The coefficients were calculated by dividing the IC_{50} values of the listed enzyme activities by the IC_{50} value (determined in the presence of 30 or 60μg of protein/ml respectively) of the calmodulin-dependent erythrocyte Ca^{2+}-transporting ATPase activity. Abbreviations: SR, sarcoplasmic reticulum; SL, sarcolemma.

	IC_{50} values		Coefficient of specificity	
Enzyme	Calmidazolium (μM)	Compound 48/80 (μg/ml)	Calmidazolium	Compound 48/80
Cardiac SR Ca^{2+}-transporting ATPase	2.1	16	6	19
Skeletal-muscle SR Ca^{2+}-transporting ATPase	2.9	80	8.3	94
Cardiac SL Na^+/K^+-transporting ATPase	15	>300	43	>353
Skeletal-muscle SR Mg^{2+}-dependent ATPase	3.3	⩾300	9.5	⩾353
Erythrocyte Mg^{2+}-dependent ATPase	20	⩾300	57	⩾353
Erythrocyte Ca^{2+}-transporting ATPase				
Basal	9	⩾300	26	⩾353
Oleic acid-activated*	10	7	25	8.2
Proteolysis-activated*	4	27	7	32

respectively (Table 1). In contrast, compound 48/80 did not significantly inhibit the activity of both Mg^{2+}-dependent ATPases in the investigated concentration range (Table 1).

The results of investigations to determine the potency of calmidazolium and compound 48/80 in antagonizing the stimulation of erythrocyte Ca^{2+}-transporting ATPase induced by different activating treatments are shown in Table 1. Half-maximal inhibition of the activity stimulated by oleic acid or mild tryptic digestion required 25 and 7 times higher concentrations of calmidazolium respectively, compared with the calmodulin-dependent fraction of the ATPase activity when assayed under identical conditions. Compound 48/80 shares with calmidazolium the property of antagonizing preferentially the calmodulin-induced stimulation of Ca^{2+}-transporting ATPase. Half-maximal inhibition of the activating effects of calmodulin, oleic acid or limited proteolysis occurred at concentrations of 0.85, 7 and 27μg of compound 48/80/ml respectively.

Discussion

In various studies evidence has been presented that putative calmodulin antagonists not only bind to calmodulin but may have more targets, as pointed out in the introduction. Because of the unspecific effects of so-called calmodulin inhibitors many studies are questionable when inhibition of some process by a presumed calmodulin antagonist is taken as evidence for a regulatory role of calmodulin in that process. Therefore it is desirable to have a more specific tool elucidating the possible involvement of calmodulin in biological processes.

In the present study the two powerful calmodulin antagonists calmidazolium and compound 48/80, were compared with respect to their specificity in antagonizing effects mediated by calmodulin. The results presented here demonstrate the greater specificity of compound 48/80 over calmidazolium in inhibiting calmodulin-dependent, as opposed to calmodulin-independent, enzyme activities. This is summarized in Table 1, which provides a list of specificity coefficients of the two calmodulin antagonists for several enzyme activities. The coefficients represent the factors by which the IC_{50} values obtained for the mentioned enzyme activities were found to be higher as compared with the IC_{50} value for the calmodulin-dependent erythrocyte Ca^{2+}-transporting ATPase activity.

Compound 48/80 was found to be superior to calmidazolium in the following ways. (1) This substance exclusively antagonized the calmodulin-induced stimulation of phosphodiesterase and erythrocyte Ca^{2+}-transporting ATPase without suppression of the basal activity of these enzymes. Calmidazolium and other calmodulin antagonists also affect the basal activity of calmodulin-dependent enzymes (Levin & Weiss, 1976; Kobayashi

et al., 1979; Gietzen *et al.*, 1980, 1981, 1983). (2) Compound 48/80 altered calmodulin-independent Ca^{2+}-transporting ATPases far less than calmidazolium (see Table 1) or phenothiazines (Balzer *et al.*, 1968; K. Gietzen, P. Adamczyk-Engelmann, A. Wüthrich, A. Konstantinova & H. Bader, unpublished work) did. (3) Ca^{2+}–calmodulin-independent enzymes, like Mg^{2+}-dependent ATPases from different tissues and Na^+/K^+-transporting ATPase of cardiac sarcolemma were not, or only slightly, affected by compound 48/80 (Table 1). Calmidazolium (Table 1) and phenothiazines (Luthra, 1982; K. Gietzen, P. Adamczyk-Engelmann, A. Wüthrich, A. Konstantinova & H. Bader, unpublished work) also inhibited these enzymes, although at higher concentrations than those needed for calmodulin antagonism. (4) Antagonism of proteolysis-induced erythrocyte Ca^{2+}-transporting ATPase activity required only a seven times higher concentration of calmidazolium but a 32-fold higher concentration of compound 48/80 as compared with inhibition of the calmodulin-stimulated ATPase activity by the respective drug (Table 1). In contrast, trifluoperazine and penfluridol were shown to be equipotent in antagonizing both the calmodulin- and proteolysis-stimulated activity of erythrocyte Ca^{2+}-transporting ATPase (Gietzen *et al.*, 1982*a*).

Inhibition of basal activity of calmodulin-regulated enzymes and antagonism of the activity of calmodulin-independent enzymes by calmidazolium and other putative calmodulin antagonists, as mentioned in points (1)–(3) of the preceding paragraph, might be a direct effect on the target enzyme and/or a consequence of perturbation of the lipid environment in the case of membrane-integral enzymes (Raess & Vincenzi, 1980; Au, 1981; Luthra, 1982). The high specificity of compound 48/80 may be determined by the polymeric structure of compound 48/80, as was suggested by Gietzen *et al.* (1983). It was proposed that this structure may hinder incorporation of the agent into biological membranes and thus perturbation of the lipid environment of membrane-bound enzymes may be less favourable.

In addition to its high specificity compound 48/80 displayed a high potency to antagonize the calmodulin-dependent fraction of phosphodiesterase and erythrocyte Ca^{2+}-transporting ATPase activity. Experiments aimed at the identification of the active constituents of compound 48/80 indicate an average M_r of approx. 1000 for these species (P. Adamczyk-Engelmann & K. Gietzen, unpublished work). Thus IC_{50} values of this calmodulin antagonist for phosphodiesterase and Ca^{2+}-transporting ATPase, expressed in terms of molarity, are comparable with those of calmidazolium.

In summary, it may be concluded that compound 48/80 is an outstanding calmodulin inhibitor in that this substance combines high potency and high specificity in antagonizing preferentially calmodulin-mediated enzyme activities. Therefore compound 48/80 is proposed to be a much more specific tool for studying the involvement of calmodulin in biological functions than the existing substances.

For the gift of skeletal-muscle sarcoplasmic-reticulum Ca^{2+}-transporting ATPase, cardiac sarcolemma Na^+/K^+-transporting ATPase and cardiac sarcoplasmic-reticulum Ca^{2+}-transporting ATPase I thank Dr. S. Fleischer, Vanderbilt University, Nashville, TN, U.S.A., Dr. P. Rosenbeiger, University of München, München, Germany, and Dr. Dagmar Hartweg, University of Ulm, respectively. I also thank Professor H. Bader for his encouragement to perform this study. I am grateful to Ms. Angela Mansard-Glogger and Ms. Anka Konstantinova for their excellent technical assistance. This work was supported by a grant from the Deutsche Forschungsgemeinschaft.

References

Au, K. S. (1981) *Gen. Pharmacol.* **12**, 285–290

Balzer, H., Makinose, M. & Hasselbach, W. (1968) *Arch. Pharmacol. Exp. Pathol.* **260**, 444–455

Butcher, R. W. & Sutherland, E. W. (1962) *J. Biol. Chem.* **237**, 1244–1250

Caroni, P., Reinlib, L. & Carafoli, E. (1980) *Proc. Natl. Acad. Sci. U.S.A.* **77**, 6354–6358

Cheung, W. Y. (1980) *Science* **207**, 19–27

Gietzen, K. & Bader, H. (1980) *IRCS Med. Sci.* **8**, 396–397

Gietzen, K. & Kolandt, J. (1982) *Biochem. J.* **207**, 155–159

Gietzen, K., Mansard, A. & Bader, H. (1980) *Biochem. Biophys. Res. Commun.* **94**, 674–681

Gietzen, K., Wüthrich, A. & Bader, H. (1981) *Biochem. Biophys. Res. Commun.* **101**, 418–425

Gietzen, K., Sadorf, I. & Bader, H. (1982*a*) *Biochem. J.* **207**, 541–548

Gietzen, K., Wüthrich, A. & Bader, H. (1982*b*) *Mol. Pharmacol.* **22**, 413–420

Gietzen, K., Sanchez-Delgado, E. & Bader, H. (1983) *IRCS Med. Sci.* **11**, 12–13

Jones, L. R., Besch, H. R., Jr., Fleming, J. W., McConnaughey, M. M. & Watanabe, A. M. (1979) *J. Biol. Chem.* **254**, 530–539

Kakiuchi, S., Sobue, K., Yamazaki, R., Kambayashi, J., Sakon, M. & Kosaki, G. (1981) *FEBS Lett.* **126**, 203–207

Klee, C. B., Crouch, T. H. & Richman, P. G. (1980) *Annu. Rev. Biochem.* **49**, 489–515

Kobayashi, R., Tawata, M. & Hidaka, H. (1979) *Biochem. Biophys. Res. Commun.* **88**, 1037–1045

Lanzetta, P. A., Alvarez, L. J., Reinach, P. S. & Candia, O. A. (1979) *Anal. Biochem.* **100**, 95–97

Levin, R. M. & Weiss, B. (1976) *Mol. Pharmacol.* **12**, 581–589

Luthra, M. (1982) *Biochim. Biophys. Acta* **692**, 271–277

Means, A. R. & Dedman, J. R. (1980) *Nature (London)* **285**, 73–77

Meissner, G., Conner, G. E. & Fleischer, S. (1973) *Biochim. Biophys. Acta* **298**, 246–269

Raess, B. U. & Vincenzi, F. F. (1980) *Mol. Pharmacol.* **18**, 253–258

Stewart, D. J. (1974) *Anal. Biochem.* **62**, 349–364

Suko, J. & Hasselbach, W. (1976) *Eur. J. Biochem.* **64**, 123–130

Van Belle, H. (1981) *Cell Calcium* **2**, 483–494

Volpi, M., Sha'Afi, R. I. & Feinstein, M. B. (1981) *Mol. Pharmacol.* **20**, 363–370

Wang, J. H. & Desai, R. (1977) *J. Biol. Chem.* **252**, 4175–4184

Watanabe, K., Williams, E. F., Law, J. S. & West, W. L. (1979) *Experientia* **35**, 1487–1489

Weiss, B., Prozialeck, W. & Cimino, M. (1980) *Ann. N.Y. Acad. Sci.* **356**, 319–345

Wolf, H. U. (1973) *Experientia* **29**, 241–249

Wolff, D. J. & Brostrom, C. O. (1976) *Arch. Biochem. Biophys.* **173**, 720–731

EXHIBIT D

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Calmidazolium and Compound 48/80 Inhibit Calmodulin-dependent Protein Phosphorylation and ATP-dependent Ca^{2+} Uptake but Not Ca^{2+}-ATPase Activity in Skeletal Muscle Sarcoplasmic Reticulum*

(Received for publication, November 14, 1983)

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Two specific calmodulin antagonists, compound 48/80 and calmidazolium, at concentrations of 10–20 μg/ml and 10–20 μM, respectively, inhibited Ca^{2+} uptake in skeletal muscle sarcoplasmic reticulum vesicles without affecting Ca^{2+}-ATPase activity. The drugs also inhibited the calmodulin-dependent phosphorylation of 85,000-, 60,000-, and 20,000-dalton proteins, but not the calmodulin-independent phosphorylation of other sarcoplasmic reticulum proteins. The inhibition of phosphorylation of the 60,000-dalton protein closely paralleled the inhibition of Ca^{2+} uptake. Neither drug affected the passive permeability of the sarcoplasmic reticulum membrane at concentrations up to 5 times the inhibitory dose, and neither drug inhibited Ca^{2+} uptake into liposomes reconstituted with the purified Ca^{2+}-ATPase. However, calmodulin-dependent reconstitution of Ca^{2+} uptake in EGTA-extracted sarcoplasmic reticulum vesicles was inhibited by 48/80.

The results of this study suggest that the calmodulin-dependent phosphorylation system plays a functional role in the coupling of ATP hydrolysis and Ca^{2+} accumulation, perhaps through regulation of Ca^{2+} release channels in the sarcoplasmic reticulum membrane. Perturbation of phosphorylation by 48/80 and calmidazolium may lead to enhanced Ca^{2+} release, thereby diminishing Ca^{2+} accumulation without affecting the Ca^{2+} uptake mechanism.

The role of calmodulin as a second messenger in the regulation of Ca^{2+}-dependent processes in cell function has been studied extensively (1). In the presence of Ca^{2+}, calmodulin regulates a multitude of enzymes, including phosphodiesterase (2–4), adenylate cyclase (5), myosin light chain kinase (6), and the (Ca^{2+} + Mg^{2+})-dependent ATPase of plasma membrane preparations from erythrocytes (7) and heart (8, 9). This (Ca^{2+} + Mg^{2+})-ATPase is involved in the active extrusion of Ca^{2+} from the cytoplasm and the regulation of cytosolic Ca^{2+} levels. Calmodulin activation of this enzyme results in an increase in V_{max} and a decrease in the K_m for Ca^{2+}.

Calmodulin has also been shown to stimulate Ca^{2+} uptake into cardiac sarcoplasmic reticulum (10) through an increase in coupled Ca^{2+}-dependent ATPase activity. Stimulation results from a cascade in which a calmodulin-dependent protein kinase phosphorylates a 22,000-dalton subunit of the ATPase, designated phospholamban (11). Phospholamban can also be phosphorylated by a cAMP-dependent protein kinase (12). Calmodulin stimulation of Ca^{2+} transport in cardiac sarcoplasmic reticulum may also contribute to the lowering of cytosolic Ca^{2+} since it lowers the K_m of the Ca^{2+} ATPase for Ca^{2+}.

Calmodulin has not yet been shown to affect Ca^{2+} uptake and Ca^{2+}-dependent ATPase activity in skeletal muscle sarcoplasmic reticulum (13). However, calmodulin is a component of isolated sarcoplasmic reticulum (14, 15), and it has been localized, by immunocytochemical techniques, in the sarcoplasmic reticulum (16). Moreover, the presence of a calmodulin-dependent protein kinase system in skeletal muscle sarcoplasmic reticulum, which results in the phosphorylation of three sarcoplasmic reticulum proteins, has been reported (13–15).

In previous studies of the role of calmodulin, we noted that trifluoperazine would inhibit Ca^{2+} accumulation in the sarcoplasmic reticulum (14). At slightly higher concentrations, however, it inhibited the Ca^{2+}-ATPase. Therefore, we could not conclude that there was an uncoupling of Ca^{2+} uptake and Ca^{2+}-ATPase due to calmodulin antagonism. Recently, Gietzen *et al.* (17) and Van Belle (18) reported that compound 48/80 and calmidazolium, respectively, are potent and specific inhibitors of calmodulin-mediated reactions. We have used 48/80 and calmidazolium to inhibit the membrane-bound calmodulin activity in order to investigate the role of this protein in the function of the sarcoplasmic reticulum. In this paper, we report that compound 48/80 and calmidazolium are able to uncouple Ca^{2+} uptake and Ca^{2+}-ATPase activity at concentrations where they specifically inhibit the calmodulin-dependent phosphorylation system of the sarcoplasmic reticulum.

EXPERIMENTAL PROCEDURES

Materials—[γ-^{32}P]ATP and $^{45}Ca^{2+}$ were obtained from New England Nuclear. Compound 48/80 was purchased from Sigma. Calmodulin was prepared from bovine cerebral cortex by the method of Teo *et al.* (3). Sodium dodecyl sulfate, acrylamide, *N,N'*-methylenebisacrylamide, 2-mercaptoethanol, and TEMED[1] were purchased from Bio-Rad. Calmidazolium was a gift from Dr. Herman Van Belle, Janssen Pharmaceutica, Belgium.

Preparation of Sarcoplasmic Reticulum Vesicles—Sarcoplasmic reticulum vesicles were prepared from rabbit skeletal muscle according

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[1] The abbreviations used are: TEMED, *N,N,N',N'*-tetramethylethylene diamine; EGTA, ethylene glycol bis(β-amino ethyl ether)-*N,N,N',N'*-tetraacetic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; PIPES, 1,4-piperazinediethanesulfonic acid.

to the method of Campbell and MacLennan (14). Sarcoplasmic reticulum vesicles were further fractionated into light and heavy fractions by linear sucrose density gradient centrifugation as described previously (19). Analysis of the protein composition of the two fractions by sodium dodecyl sulfate-polyacrylamide gel electrophoresis confirmed the reported differences (20). Purified (R_3C) and partially purified (R_2) ATPase were obtained as described by MacLennan (21).

Removal of Endogenous Calmodulin from Sarcoplasmic Reticulum—Sarcoplasmic reticulum vesicles at 10 mg of protein/ml were incubated for 15 min at 0 °C in a solution containing 0.25 M sucrose, 10 mM Tris-HCl, 1 mM histidine, pH 8.0 (buffer A), containing 1 mM EGTA, and then centrifuged for 15 min at 105,000 × *g* (17). This procedure was repeated twice more, and the final pellet was suspended in buffer A at a concentration of 20 mg/ml.

Calmodulin-dependent Reconstitution of Ca^{2+} Uptake in Sarcoplasmic Reticulum Vesicles—Sarcoplasmic reticulum vesicles, extracted with EGTA to remove endogenous calmodulin as described above, were incubated at room temperature for 10 min with 0.6 μM calmodulin in the presence of 1 mM $CaCl_2$, 5 mM $MgCl_2$, and 5 mM ATP at a protein concentration of 10 mg/ml. Ca^{2+} uptake was then initiated by diluting 400 μg of reconstituted protein in 2 ml of Ca^{2+} uptake buffer which contained 5 mM ATP, 5 mM $MgCl_2$, 100 mM KCl, 0.5 mM EGTA, 0.5 mM $^{45}CaCl_2$, 5 mM oxalate, and 20 mM histidine, pH 7.0. Ca^{2+} uptake was assayed at 30-s intervals.

Reconstitution of Ca^{2+}-ATPase Vesicles—The cholate dialysis method was used (22). A suspension of 30 mM phospholipids in 1.6% sodium cholate and 0.4 M potassium phosphate, pH 7.5, was briefly sonicated in a water bath sonicator. Purified ATPase (R_3C) or partially purified ATPase (R_2) was added in the ratio of 1 mg of protein/ml in the presence of 1.5 mg of deoxycholate, and the mixture was dialyzed at 4 °C for 16 h against 500 ml of 0.4 M potassium phosphate buffer, pH 7.5.

Measurement of Permeability of Sarcoplasmic Reticulum Vesicles—Sarcoplasmic reticulum vesicles were incubated at 10–15 mg/ml for 16 h in a medium containing 150 mM KCl, 5 mM $MgCl_2$, 20 mM MOPS, pH 7.0, and 5 mM $^{45}CaCl_2$ to achieve passive loading. ^{45}Ca efflux was measured with the filtration method after a 20-fold dilution of the suspension in 150 mM KCl, 5 mM $MgCl_2$, and 20 mM MOPS, pH 7.0.

Calmodulin-dependent Phosphorylation of Sarcoplasmic Reticulum—Phosphorylation was carried out at 30 °C as described by Campbell and MacLennan (14) in 100 μl of kinase buffer consisting of 50 mM PIPES, pH 7.0, 10 mM $MgCl_2$, 10 mM NaF, 0.2 mM EGTA, 0.5 mM $CaCl_2$, 10 μM [γ-^{32}P]ATP (2000 cpm/pmol), and 50–100 μg of sarcoplasmic reticulum protein in the presence or absence of 0.6 μM calmodulin. After 1 min, the reaction was terminated by the addition of 50 μl of a solution containing 6% sodium dodecyl sulfate, 188 mM Tris-HCl, pH 6.8, 3% 2-mercaptoethanol, 1 mM EGTA, 30% glycerol, and 0.001% bromphenol blue. Samples were boiled at 100 °C for 3 min and applied quantitatively to sodium dodecyl sulfate-polyacrylamide slab gels. Radioactive bands were localized by autoradiography and cut out of the dried gel, and the radioactivity was quantitated by liquid scintillation counting.

Gel Electrophoresis and Autoradiography—Sodium dodecyl sulfate-polyacrylamide slab gel electrophoresis was performed using the discontinuous buffer system of Laemmli (23) in 1.5-mm thick slab gels containing 10% acrylamide. Autoradiography of dried slab gels were performed using Kodak X-Omat film and a DuPont Cronex Lightning Plus enhancing screen.

Assays—Active Ca^{2+} uptake was assayed by the Millipore filtration method as described previously (24). Ca^{2+}-ATPase activity was measured as described previously (21). Protein concentration was determined according to Lowry *et al.* (25). Phosphodiesterase activity was determined as described in Ref. 26.

RESULTS

Effect of 48/80 and Calmidazolium on Ca^{2+} Uptake in Sarcoplasmic Reticulum Vesicles—One of the ways to study the physiological role of calmodulin in sarcoplasmic reticulum membranes is to inhibit the activity of this protein selectively, using specific calmodulin antagonists. Calmidazolium (18) is a potent calmodulin antagonist, devoid of affinity toward other receptors. Gietzen *et al.* (17) have also reported that 48/80 is a powerful and specific inhibitor of calmodulin-stimulated Ca^{2+} uptake in the erythrocyte membrane. Fig. 1 indicates that compound 48/80 inhibits calmodulin-activated phosphodiesterase activity, without any effect on the basal activity of the enzyme.

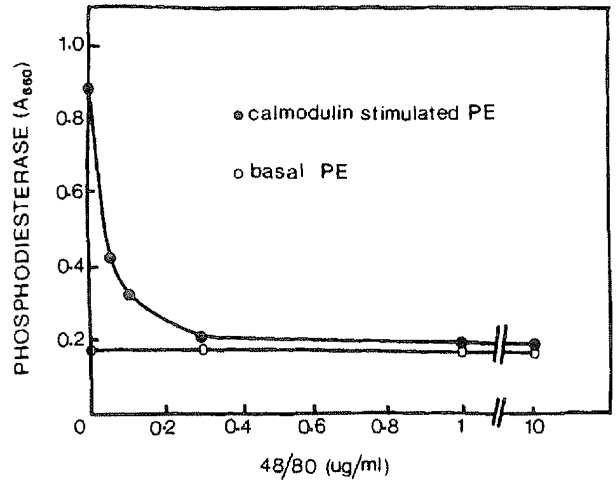

FIG. 1. **Effect of 48/80 on calmodulin-stimulated and basal phosphodiesterase activity.** Phosphodiesterase (*PE*) activity was measured in a reaction mixture containing: 40 mM Tris-HCl, pH 7.5, 40 mM imidazole, 5 mM magnesium acetate, 0.1 mM $CaCl_2$, 1.2 mM cAMP, and various amounts of 48/80 in the absence (○) and presence (●) of 0.3 μM calmodulin.

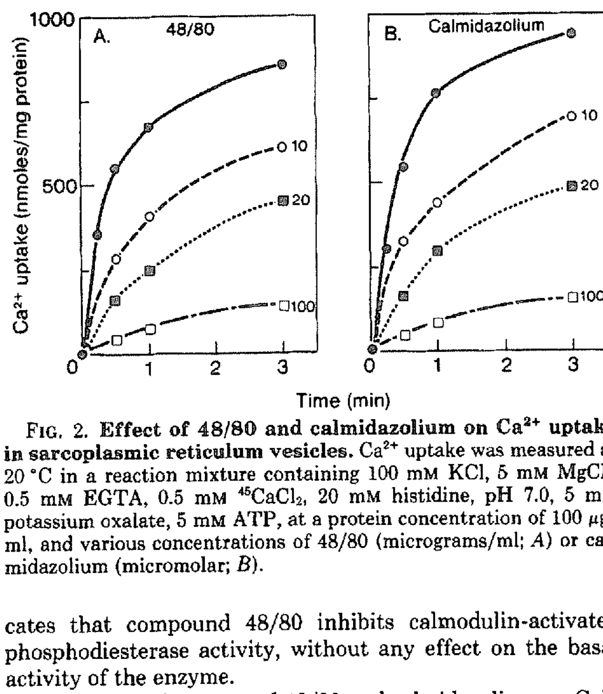

FIG. 2. **Effect of 48/80 and calmidazolium on Ca^{2+} uptake in sarcoplasmic reticulum vesicles.** Ca^{2+} uptake was measured at 20 °C in a reaction mixture containing 100 mM KCl, 5 mM $MgCl_2$, 0.5 mM EGTA, 0.5 mM $^{45}CaCl_2$, 20 mM histidine, pH 7.0, 5 mM potassium oxalate, 5 mM ATP, at a protein concentration of 100 μg/ml, and various concentrations of 48/80 (micrograms/ml; *A*) or calmidazolium (micromolar; *B*).

The effects of compound 48/80 and calmidazolium on Ca^{2+} uptake by the sarcoplasmic reticulum are shown in Fig. 2. Both drugs inhibited Ca^{2+} uptake rather potently. The concentration of drug for 50% inhibition (I_{50}) determined from initial Ca^{2+} uptake rates varied between 10 and 20 μM from preparation to preparation, possibly reflecting the amount of calmodulin that remained associated with the sarcoplasmic reticulum membrane during isolation. When the sarcoplasmic reticulum was fractionated into light and heavy fractions using sucrose density gradients, the inhibition of Ca^{2+} uptake by 48/80 was similar in each fraction (data not shown).

The observed inhibition of Ca^{2+} uptake could have been due to inhibition of Ca^{2+}-ATPase, the enzyme responsible for Ca^{2+} pumping. Fig. 3 shows that Ca^{2+}-ATPase activity was essentially unaltered at drug concentrations where Ca^{2+} up-

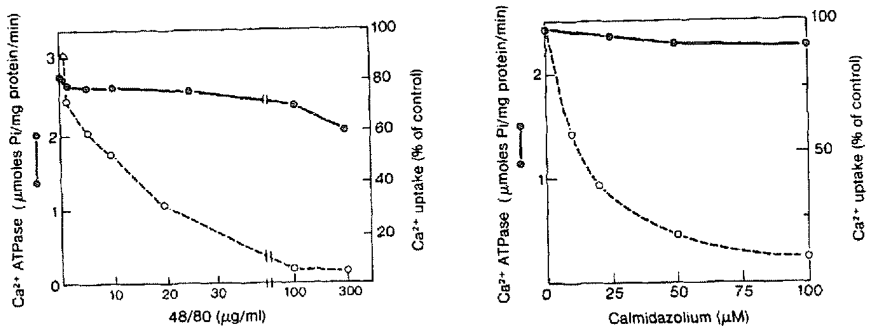

FIG. 3. **Effect of 48/80 and calmidazolium on Ca^{2+}-ATPase and Ca^{2+} uptake activity of sarcoplasmic reticulum vesicles.** Ca^{2+}-ATPase activity was measured under identical conditions to those used for Ca^{2+} uptake as described in the legend to Fig. 2, except that [γ-^{32}P]ATP and unlabeled $CaCl_2$ were used. ●, Ca^{2+} ATPase; ○, Ca^{2+} uptake.

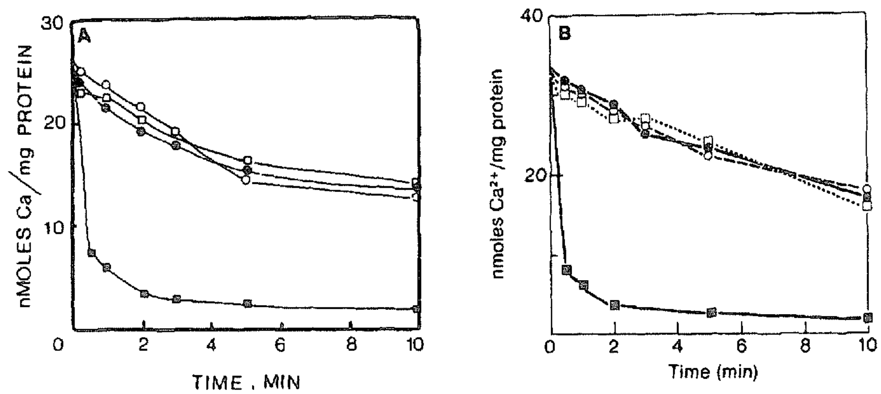

FIG. 4. **Effect of 48/80 and calmidazolium on the passive permeability of sarcoplasmic reticulum vesicles to $^{45}CaCl_2$.** Sarcoplasmic reticulum vesicles were passively loaded with $^{45}CaCl_2$ as described under "Experimental Procedures". The Millipore filtration technique was used to determine [^{45}Ca] efflux in the presence and absence of: *A*, 0 (○), 25 (●), and 50 (□) μg/ml of 48/80 or 5 μg/ml of A23187 (▩); *B*, 0 (○), 25 (●), and 50 (□) μM calmidazolium or 5 μg/ml of A23187 (▩).

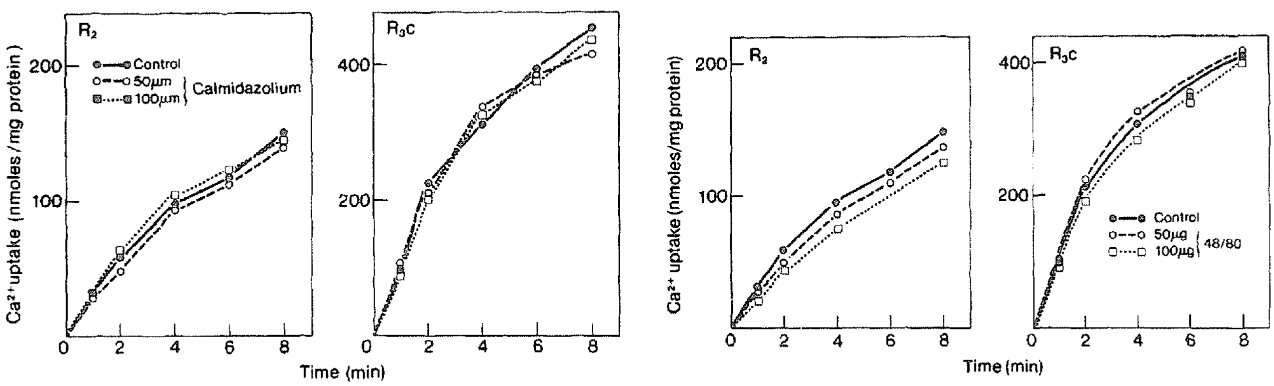

FIG. 5. **48/80 and calmidazolium have no effect on Ca^{2+} uptake in liposomes reconstituted with the partially purified and purified Ca^{2+}-ATPase.** Proteoliposomes were prepared as described under "Experimental Procedures". Ca^{2+} uptake by proteoliposomes was assayed as described in the legend to Fig. 2. Where indicated, the assay medium contained protein at a concentration of 50 μg/ml, 48/80 at 50 or 100 μg/ml, and calmidazolium at 50 or 100 μM. R_2, partially purified ATPase-containing liposomes; R_3C, purified ATPase-containing liposomes (21).

take was potently inhibited. Both 48/80 and calmidazolium appeared to be equally effective in uncoupling Ca^{2+}-ATPase from Ca^{2+} uptake.

In order to rule out a nonspecific interaction of 48/80 and calmidazolium with the sarcoplasmic reticulum, leading to a change in Ca^{2+} permeability and thereby to a decrease in Ca^{2+} uptake, the effect of the drugs on Ca^{2+} efflux from passively loaded vesicles was studied. Fig. 4 shows that drug concentrations as high as 50 μM induced no change in the passive permeability of the sarcoplasmic reticulum membranes to Ca^{2+}. By comparison, the Ca^{2+} ionophore A23187 dramatically increased the permeability of the membranes to Ca^{2+}.

Effect of 48/80 and Calmidazolium on ATPase in Reconstituted Liposomes—The ability of 48/80 and calmidazolium to inhibit Ca^{2+} accumulation without affecting Ca^{2+}-ATPase activity or the passive permeability of the sarcoplasmic reticulum membrane to Ca^{2+} suggested either that the compounds were uncoupling the inward movement of Ca^{2+} from the ATP hydrolytic activity of the Ca^{2+}-ATPase or that these drugs were activating a specific Ca^{2+} release mechanism so that the accumulated Ca^{2+} was immediately released. Since Ca^{2+} accumulation is believed to be an integral part of the enzymatic mechanism of ATP hydrolysis (27), it seems unlikely that enzymatic uncoupling would occur. Fig. 5 demonstrates that 48/80 and calmidazolium had no effect on Ca^{2+} accumulation in asolectin vesicles reconstituted with the partially purified ATPase (R_2) and fully purified ATPase (R_3C). These results also indicate that the inhibition of Ca^{2+} accumulation by 48/80 and calmidazolium is not due to a nonspecific interaction with the lipid bilayer or the Ca^{2+} pumping mechanism, but

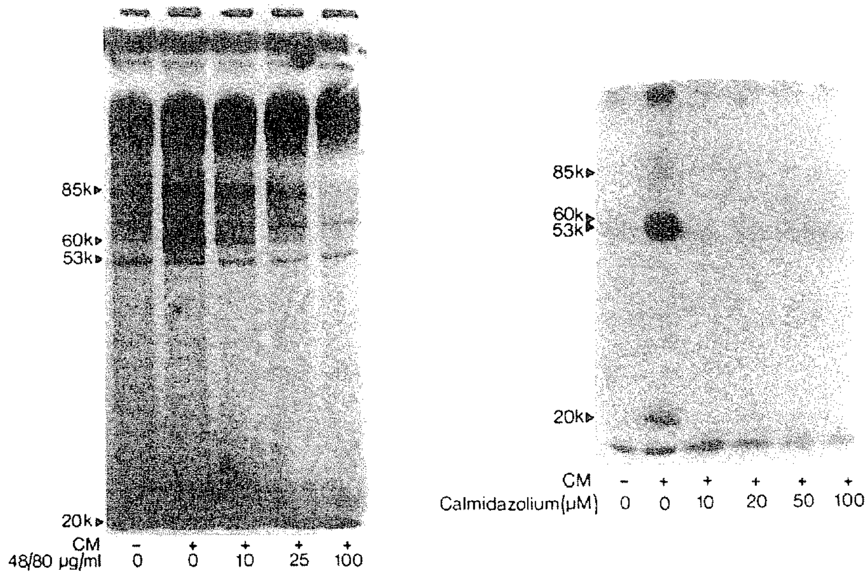

FIG. 6. **Specificity of 48/80 and calmidazolium for the calmodulin-dependent protein kinase system of sarcoplasmic reticulum.** EGTA-washed sarcoplasmic reticulum vesicles were phosphorylated in the absence and presence of calmodulin (*CM*) and various concentrations of 48/80 and calmidazolium as indicated.

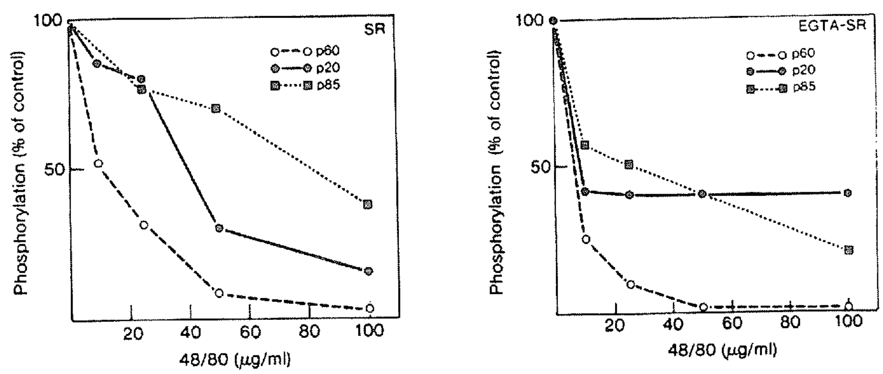

FIG. 7. **Inhibition by 48/80 of ^{32}P incorporation into various proteins phosphorylated by the calmodulin-dependent protein kinase system of sarcoplasmic reticulum.** Phosphorylation of sarcoplasmic reticulum (*SR*) and EGTA-washed sarcoplasmic reticulum was carried out as described under "Experimental Procedures" in the presence of 0.6 μM calmodulin and various concentrations of 48/80. Controls were run in the absence of calmodulin. The radioactive 85,000-, 60,000- and 20,000-dalton protein bands were localized by autoradiography, cut from the gel, and quantitated by lipid scintillation counting. The percentage phosphorylation compared with maximum phosphorylation in the absence of 48/80 is plotted against increasing 48/80 concentrations in the phosphorylation buffer.

must inhibit some specific receptor system which is absent in the partially purified and purified ATPase preparations.

Calmodulin in Sarcoplasmic Reticulum As a Site of Action of 48/80 and Calmidazolium—Calmodulin has been found to be intimately associated with the sarcoplasmic reticulum membrane (14, 15) and to stimulate a protein kinase activity, intrinsic to the sarcoplasmic reticulum membrane, resulting in the phosphorylation of proteins of $M_r = 85{,}000$, 60,000, and 20,000. Since 48/80 and calmidazolium have been reported to antagonize calmodulin specifically, the effects of these two compounds on the calmodulin-dependent protein kinase activity of sarcoplasmic reticulum were investigated.

Fig. 6 shows that activation of a calmodulin-dependent protein kinase in the presence of Ca^{2+} and calmodulin results in the phosphorylation of three proteins of $M_r = 85{,}000$, 60,000, and 20,000 in sarcoplasmic reticulum vesicles. A calmodulin-independent protein kinase was also activated under the reaction conditions resulting in the phosphorylation of a protein of $M_r = 53{,}000$. Fig. 6 also shows that 48/80 and calmidazolium potently and specifically inhibited the phosphorylation due to the calmodulin-dependent protein kinase but that they did not alter the phosphorylation due to the calmodulin-independent protein kinase. A component of M_r greater than 200,000 phosphorylated in the presence of calmodulin and sensitive to calmidazolium and 48/80, was probably an aggregate of the calmodulin-dependent phosphoproteins. Quantitation of the inhibition of ^{32}P incorporation into proteins of $M_r = 85{,}000$, 60,000, and 20,000 by 48/80 is shown in Fig. 7. The incorporation of ^{32}P into the 60,000-dalton protein was more sensitive than incorporation into the 85,000- and the 20,000-dalton proteins (Fig. 7). The inhibition of calmodulin-dependent ^{32}P incorporation into the 60,000-dalton proteins, in both sarcoplasmic reticulum and EGTA-extracted sarcoplasmic reticulum, closely paralleled the inhibition of Ca^{2+} uptake in sarcoplasmic reticulum.

48/80 Inhibition of Calmodulin-dependent Reconstitution of Ca^{2+} Uptake in Sarcoplasmic Reticulum—As reported previously (28), removal of calmodulin from sarcoplasmic reticulum by EGTA extraction resulted in a marked decrease in Ca^{2+} uptake (Table I). Ca^{2+} uptake was partially restored

TABLE I

Inhibition by 48/80 of calmodulin-dependent reconstitution of Ca^{2+} uptake in EGTA-washed sarcoplasmic reticulum

Sarcoplasmic reticulum vesicles were extracted with EGTA to remove endogenous calmodulin as described under "Experimental Procedures". Ca^{2+} uptake in the EGTA-extracted sarcoplasmic reticulum pellet was measured as described in the legend to Fig. 2 except that 100 μg/ml of protein were preincubated with calmodulin and various cofactors, as indicated, prior to Ca^{2+} uptake measurement. The concentrations of various cofactors were: $CaCl_2$, 1 mM; calmodulin (CaM), 0.6 μM; $MgCl_2$, 5 mM; ATP, 5 mM; and 48/80, 5 or 10 μg/ml.

Additions to preincubation medium	Ca^{2+} uptake
	nmol/mg/min
None	32
48/80 (10 μg)	31
CaM, Ca^{2+}, Mg^{2+}, ATP	104
CaM, Ca^{2+}, Mg^{2+}, ATP, 48/80 (5 μg)	82
CaM, Ca^{2+}, Mg^{2+}, ATP, 48/80 (10 μg)	54

upon the addition of exogenous calmodulin under conditions which are known to activate the phosphorylation mechanism (14). However, the calmodulin-dependent reconstitution of Ca^{2+} transport was inhibited almost 70% by 48/80 at 10 μg/ml. This observation supports the view that 48/80 is interacting with calmodulin and inhibiting the calmodulin-dependent phosphorylation reaction, thereby resulting in decreased Ca^{2+} uptake.

DISCUSSION

In this study, we have found that compound 48/80 and calmidazolium are powerful and specific inhibitors of the calmodulin-dependent protein kinase system of the sarcoplasmic reticulum. Ca^{2+}-ATPase activity of the sarcoplasmic reticulum was not affected by the drugs, but Ca^{2+} accumulation was potently inhibited in the same concentration range where calmodulin-stimulated kinase activity was inhibited. Calmidazolium and compound 48/80 did not affect the passive permeability of the sarcoplasmic reticulum to Ca^{2+}. Ca^{2+} accumulation into asolectin vesicles reconstituted with the purified Ca^{2+}-ATPase was not affected by either compound 48/80 or calmidazolium, demonstrating that these two drugs were not disrupting the lipid bilayer and were not directly inhibitory to the Ca^{2+} pumping function of the Ca^{2+}-ATPase. Our results also demonstrate that 48/80 antagonizes the calmodulin-dependent reconstitution of Ca^{2+} uptake into EGTA-extracted sarcoplasmic reticulum.

A role for calmodulin in the coupling of Ca^{2+} uptake and Ca^{2+}-ATPase activity is suggested by these studies. Other observations also support a role for calmodulin in the Ca^{2+} accumulation process. EGTA extraction, which removes calmodulin and lowers calmodulin-dependent protein phosphorylation, leads to diminished Ca^{2+} uptake, enhanced ATP hydrolysis, and increased membrane permeability (28, 29). Elevation of pH above 6.8 also diminishes Ca^{2+} accumulation, enhances ATPase activity, and diminishes calmodulin-dependent phosphorylation of the 60,000-dalton protein (14). In reconstituted systems containing the purified ATPase, however, Ca^{2+} accumulation proceeds nicely at pH 7.5 (30).

Similar observations have been made with tetraphenylboron (31), but in this case, the effect is better understood. Tetraphenylboron inhibits Ca^{2+} accumulation but enhances Ca^{2+}-ATPase. It also induces Ca^{2+} release from sarcoplasmic reticulum perhaps by altering surface charges that affect Ca^{2+} release channels (31). It does not release Ca^{2+} from reconstituted systems containing the Ca^{2+}-ATPase. This set of observations suggests that there are Ca^{2+} release sites in the sarcoplasmic reticulum that are absent from membranes reconstituted with the Ca^{2+}-ATPase. They are sensitive to tetraphenylboron and may be sensitive to calmodulin, perhaps through the medium of protein phosphorylation. We have recently proposed that the calmodulin-dependent phosphorylation systems of the sarcoplasmic reticulum might be involved in the regulation of the Ca^{2+} release channel (32).

Studies on rates of phosphorylation indicate that the 60,000-dalton protein is phosphorylated rather rapidly as compared with the 20,000-dalton protein (14, 15). Our present studies also suggest that the 60,000-dalton protein phosphorylation is most drug-sensitive. Therefore, the 60,000-dalton protein is of greatest interest in further studies. Only partial purification of the 60,000- and 20,000-dalton proteins has been achieved under nondenaturing conditions. The proteins are found to be rather hydrophobic and can only be dissolved in detergents (32). The use of calmodulin affinity columns to purify the proteins has not been successful, although the 20,000-dalton protein has been purified in acidified chloroform/methanol (15). In future studies it will be necessary to achieve purification of the proteins of interest so that their roles in Ca^{2+} release in a reconstituted system can be studied.

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REFERENCES

1. Klee, C., Crouch, T. H., and Richman, P. G. (1980) *Annu. Rev. Biochem.* **49**, 489–515
2. Cheung, W. Y. (1970) *Biochem. Biophys. Res. Commun.* **38**, 533–538
3. Teo, T. S., Wang, T. H., and Wang, T. H. (1973) *J. Biol. Chem.* **248**, 588–595
4. Kakiuchi, S., and Yamazaki, R. (1970) *Biochem. Biophys. Res. Commun.* **41**, 1104–1110
5. Brostrom, C. O., Huang, Y. C., Breckenridge, B. McL., and Wolff, D. J. (1975) *Proc. Natl. Acad. Sci. U. S. A.* **72**, 64–68
6. Dabrowska, R., Sherry, J. M. F., Aromatorio, D. K., and Hartshorne, D. J. (1978) *Biochemistry* **17**, 253–258
7. Gopinath, R. M., and Vincenzi, F. F. (1977) *Biochem. Biophys. Res. Commun.* **77**, 1203–1209
8. Tuana, B. S., Dzurba, A., Panagia, V., and Dhalla, N. S. (1981) *Biochem. Biophys. Res. Commun.* **100**, 1245–1250
9. Caroni, P., and Carafoli, E. (1981) *J. Biol. Chem.* **256**, 3263–3270
10. Katz, S., and Remtulla, M. A. (1978) *Biochem. Biophys. Res. Commun.* **83**, 1373–1379
11. Kirchberger, M. A., and Tada, M. (1976) *J. Biol. Chem.* **251**, 725–729
12. Tada, M., Kirchberger, M. A., and Katz, A. M. (1975) *J. Biol. Chem.* **250**, 2640–2647
13. Chiesi, M., and Carafoli, E. (1982) *J. Biol. Chem.* **257**, 984–991
14. Campbell, K. P., and MacLennan, D. H. (1981) *J. Biol. Chem.* **257**, 1238–1246
15. Chiesi, M., and Carafoli, E. (1983) *Biochemistry* **22**, 985–993
16. Harper, J. R., Cheung, W. Y., Wallace, R. W., Levine, S. N., and Steiner, A. L. (1980) in *Calcium and Cell Function* (Cheung, W. Y., ed) Vol. 1, pp. 273–290, Academic Press, New York
17. Gietzen, K., Adamczyk-Engelmann, P., Wuthrich, A., Konstantinova, A., and Bader, H. (1983) *Biochim. Biophys. Acta* **736**, 109–118
18. Van Belle, H. (1981) *Cell Calcium* **2**, 483–494
19. Michalak, M., Campbell, K. P., and MacLennan, D. H. (1980) *J. Biol. Chem.* **255**, 1317–1326
20. Meissner, G. (1975) *Biochim. Biophys. Acta* **389**, 51–68
21. MacLennan, D. H. (1970) *J. Biol. Chem.* **245**, 4508–4518
22. Knowles, A. F., and Racker, E. (1975) *J. Biol. Chem.* **250**, 3538–3544
23. Laemmli, U. K. (1970) *Nature (Lond.)* **227**, 680–685
24. Martonosi, A., and Feretos, R. (1964) *J. Biol. Chem.* **239**, 648–658
25. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
26. Sharma, R. K., Wang, T. H., Wirch, E., and Wang, J. H. (1980) *J. Biol. Chem.* **255**, 5916–5923
27. DeMeis, L., and Vianna, A. L. (1979) *Annu. Rev. Biochem.* **48**, 275–292
28. MacLennan, D. H. (1974) in *Myocardial Biology. Recent Advances in Studies on Cardiac Structure and Metabolism* (Dhalla, N. S., ed) Vol. 4, pp. 507–517, University Park Press, Baltimore
29. Diamond, E. M., Norton, B. K., McIntosh, D. B., and Berman, M. C. (1980) *J. Biol. Chem.* **255**, 11351–11356
30. Racker, E. (1972) *J. Biol. Chem.* **247**, 8198–8200
31. Shoshan, V., MacLennan, D. H., and Wood, D. S. (1983) *J. Biol. Chem.* **258**, 2837–2842
32. MacLennan, D. H., Campbell, K. P., Takisawa, H., and Tuana, B. S. (1984) *Adv. Cyclic Nucleotide Res.* **17**, 393–401